REAGENTS AND METHODS FOR THE DETECTION OF GENES ASSOCIATED WITH THE MAJOR HISTOCOMPATIBILITY COMPLEX OF DOMESTIC FOWL, SUCH AS CHICKEN

The invention relates to the detection of genes associated with the major histocompatibility complex (MHC) of domestic fowl, such as chicken. By virtue of this, it relates to nucleic acid molecules which make it possible to detect those genes of the MHC involved in the phenomena of resistance or susceptibility to the development of virus-induced tumours. The invention also relates to the applications of said nucleic acid molecules, particularly for the development of genotyping tests in farmed birds, particularly chickens, and for the selection of animals of interest.

Infectious viral diseases are feared by breeders because of their contagious nature which leads to substantial animal losses.

Vaccination was an effective prophylaxis until the emergence of hypervirulent strains making it necessary to identify resistant haplotypes.

Various methods have, therefore, been proposed in an attempt to select those animals which are capable of resisting such diseases and those which are, in contrast, likely to be affected.

The most routinely used methods are based on serological polymorphisms or those of the RFLP type. However, these methods do not provide accurate knowledge about the phenomenon of resistance or susceptibility to disease, particularly due to lack of discrimination with respect to genes of the B or Rfp-Y systems of the MHC.

The work of the inventors on MHC gene sequencing showed the genetic complexity of this region, which led them to take account of another type of polymorphism, namely that based on the sequence of these genes and of related regions, such as those of their promoters and of the microsatellite regions. The inventors thus developed methods for obtaining highly specific oligonucleotide molecules of the polymorphisms observed, making it possible to identify the

parts of genes, and even the sites involved in controlling resistance or susceptibility to the development of tumours.

The specific nature of these molecules with respect to a given gene of one of the systems of the MHC makes them particularly reliable discriminating tools for identifying accurately the capacity for resistance or susceptibility of the chicken studied, or of other birds, to a viral infection, and for studying at molecular level the sequences of the MHC involved.

The object of the invention is, therefore, to provide nucleic acid molecules which make it possible to detect specifically, in farmed birds and particularly in chickens, the genes associated with the MHC involved in the phenomena of resistance or susceptibility to the development of virus-induced tumours.

It also provides a method and a kit for the detection of genotypes which is easy to use on a routine basis.

The nucleic acid molecules of the invention are characterised in that they are molecules, isolated from their natural environment, of nucleic acids of genes coding for proteins involved in controlling resistance or susceptibility to the development of virus-induced tumours in farmed birds, such as those of Marek's disease in chickens, with, if necessary, the regions which are attached to them such as those of the promoter or microsatellite regions. The term gene as used in the description and claims includes these regions.

These nucleic acid molecules are characterised more particularly in that they have nucleic acid sequences of genes of the B system or of the Rfp-Y system of the MHC of farmed birds, with the exception of sequences of class II B-L genes, gene 17.5, gene 12.3 and of the class I B-FIV gene, or are capable of pairing with one of the strands of a gene capable of coding for a protein as defined above under conditions of low stringency.

The pairing under conditions of low stringency to which reference is made above is carried out at ambient temperature in a 0.1 SSC medium with washing at ambient temperature.

Class II B-L genes are described in Immunogenetics 31:179-187, 1990 and Eur. J. Immunol., 1993, 23: 1139-1145.

Gene 17.5 belongs to the gene superfamily coding for lectins and gene 12.3 to the gene family coding for proteins that bind guanine (guanine nucleotide-binding protein). This gene is described in Immunogenetics 39:221-229, 1994.

Gene 12.3 is described in P.N.A.S. USA, vol. 86, 4594-4598, June 1989, Genetics.

The class I B-FIV gene is described in Immunogenetics 31:405-409, 1990.

The invention relates, in particular, to molecules of nucleic acids corresponding to those of the sequences of one of the following genes:

. sequence of the Rfp-Y system

B-FV (figure 1); B-F VI (figure 2);

. sequence of the B system,

genomic 8.4 (figure 3); B-F I (figure 4); C121 (figure 5); DM (figure 6); TAP1 (of the beginning of exon 2 at the 3' end) (figure 7); and TAP2G (figure 8), and other genes comprised in figure 10 and continuations 1 to 35.

By studying the nucleic acid sequences of the molecules defined above, it was possible to identify accurately the blocks of polymorphisms which must be detected in order to establish a reliable and accurate genotyping.

By comparing the sequences of these blocks originating from different genes of the same haplotype or from the same gene of different haplotypes, the inventors took into consideration divergent sequences, and developed for each gene complementary oligonucleotides of these divergent sequences.

Specific primers which are discriminating with respect to a given gene of the B or Rfp-Y system are obtained in this way.

The invention relates more particularly to oligonucleotide molecules corresponding to these sequences and comprising a part of the polymorphic region of the systems of the MHC of chickens or other farmed birds.

It will be recalled that the polymorphic region may be in the gene or in a related region such as the microsatellite regions or that of the promoter.

According to one embodiment of the invention, the polymorphisms are associated with the function of the systems of the MHC.

These are thus advantageously molecules corresponding to a part of an exon. Molecules corresponding to exon 2 (1 domain) of Y-F genes in chickens may be cited by way of example. A suitable pair of primers is composed of:

Y-F VI 1 : GGCCCCGGGATGCCGCGGTTC

Y-F VI 1, R : ATCCGCTCACCGCCCTGG

According to another embodiment of the invention, the oligonucleotide molecules correspond to a part of a polymorphic region which is not associated with the function of the systems of the MHC. Preferred regions of this type are microsatellites.

Taking the gene B-FI, for example, oligonucleotide molecules that can be used for composing pairs of primers correspond to the following sequences:

B-FI : 5'CCA GCA GTC ACT GCA CAT AT 3'

B-FI, R : 5' AGG TGG AGT GCG CAA AGT T 3' ; and

12.1 : 5' AGA CGC AGC AGA ACT TGG TAA 3'

12.1 R : 5' GGA AGG AAG ACC TTG GAA 3'

With the oligonucleotide molecules defined above and those developed from known genes but according to the step of the invention, highly specific sets of primers are obtained which make it possible to determine accurately the haplotype of the animal to be studied and to detect whether it is resistant to the development of virus-induced tumours or, in contrast, likely to be affected.

The invention also relates, therefore, to a method of genotyping farmed birds, particularly chickens.

This method is characterised in that it comprises:

- amplification of a sample of nucleic acid originating from the animal to be studied using one or more pairs of primers capable of hybridising specifically with the nucleic acid of a polymorphic region of the Rfp-Y or B systems of the MHC of said birds

and

- detection of the PCR products obtained.

A simple comparison of the results obtained with a reference established beforehand makes it possible to determine rapidly the haplotype of the animal.

The sample of nucleic acid is composed particularly of genomic DNA extracted from biological material of the animal to be studied or composed of this material itself, particularly the blood of the animal. It may be, as a variant, cDNA, RNA or PNA (polypeptides nucleic acids).

The primers are developed from oligonucleotide molecules defined above and, generally speaking, from any gene (and related region) coding for a protein involved in controlling resistance or susceptibility to virus-induced tumours in farmed birds, and particularly chickens, particularly class II B-L genes, 17.5, 12.3 and class I B-FIV genes.

They are, for example, primers of microsatellite regions which make it possible to detect haplotypes of the B complex such as those developed from the gene B-FI, and mentioned above, or primers which make it possible to detect haplotypes of the Rfp-Y system, and developed from gene 17.5, such as the pair:

17.52 : CAG GAT CTG CAC TGG CCA ATA

17.5, R1 : GAA TGG CGG TGC TTC CGT GCC TGG

The PCR products are detected according to conventional methods. These methods include sequencing, electrophoresis, hybridisations with SSOP or SSCP analysis.

This method will be selected advantageously according to the nature of the polymorphism involved. Thus, in the case polymorphism of the microsatellite type, the PCR products will be detected advantageously according to their size using electrophoresis methods.

If the polymorphism concerns only a few nucleotides, or even a single nucleotide, the methods used more specifically for the purposes of differentiating the haplotypes of PCR products will be hybridisation (analysis on a membrane using specific probes of the haplotype sequences, SSOP or Sequence Specific Oligonucleotide Probe), differential migration of denatured samples (SSCP or Single Strand Conformational Polymorphism) or sequencing. Generally speaking, this latter method is preferred in view of the ease with which it is carried out.

The invention thus provides a simple and rapid method for establishing the genetic profile of a large number of animals to be studied, which makes it possible to determine the haplotypes and to select those of interest with a view to breeding.

Moreover, as it is possible to distinguish each type of gene using primers having the required specificity and to establish whether it belongs to the B or Rfp-Y system, it is possible to carry out more complete fundamental studies.

The invention also relates to a unit or kit for detecting the genotype of the chicken or other farmed bird according to the method defined above.

These units or kits are characterised in that they comprise the reagents required for carrying out at least one PCR and the detection test.

In particular, they comprise the primers for PCR, a positive indicator of the reaction, and directions for use.

The primers are in the freeze-dried form or in solution or, depending on the mode of detection, on a support. By convention, the support may be a multiwell assay plate or it make take the form of DNA chips.

The invention also relates to an experimental system which enables the resistance to the tumoral development to be studied in chicken.

It corresponds to animal lines which have genetically been screened on their CMH charateristics. Depending on said charateristics, the lines are either resistant, or susceptible with respect to virus-induced tumours ; such as Marek's disease virus. This genetic selection, performed in a first step on serological criteria, has then be seeked for on the basis of the CMH genes polymorphism study.

This is a genetic material which is completely defined on a molecular point of view, and constitutes a precious tool for studying the polymorphism of microsatellite-type-sequences.

This material, as well as the cross-product between some lines between them, was used for determining CMH microsatellites sequences which are polymorphic and for studying whether such a polymorphism can be correlated to typing data already available for said lines.

Other characteristics and advantages of the invention are explained in the examples that follow in which reference is made to figure 9 representing an electrophoresis photo of PCR products illustrating the genotyping test of the invention. It will be recalled that figures 1 to 8, already mentioned above, illustrate the gene sequences according to the invention.

Example:

Study of Rfp-Y haplotypes of chickens using microsatellite primers.

- amplification with the Expand™ High Fidelity PCR System kit

. with primers 17.5 R1/17.52

genomic DNA:	1 µg
take oligos:	0.3 µM
dNTP:	8 µl
qsp H_2O:	50 µl

add 50 µl of Mix 2 whist mixing.

Mix 2:	0.75 µl of enzyme
	10 µl of TP10X with $MgCl_2$
	qsp H_2O 50 µl

Amplification programme:

30 cycles

94 C	94 C	65 C	72 C	4 C
2'	30"	1'	1'	∞

. with BFI/BFIR, R :

genomic DNA:	1 µg
take oligos:	0.3 µM
dNTP:	8 µl
qsp H_2O 50 µl	

and add 50 µl of Mix 2 whilst mixing.

Amplification programme:

	30 cycles				
	94 C	94 C	60 C	72 C	4 C
	2'	30"	1'	1'	∞

- detection by electrophoresis on agarose gel or by sequencing.

The test was applied to 9 chicken haplotypes selected serologically for the B complex. These are haplotypes B4, B5, B7, B12, B13, B14, B15, B21 and an unknown haplotype BX.

Several individuals of the same type were studied for B12 (6 individuals), B13 (3 individuals), B14 (4 individuals), B21 (4 individuals) and a single individual for the other haplotypes.

Figure 9 provides a photo of electrophoresis on 1% agarose gel of the PCR products obtained after the amplification stage.

Slopes 1 and 27 correspond to the size markers and slopes (2 to 25) to the PCR products of the following haplotypes: slope 2: B4; slope 4: B5; slope 5: B7; slopes 6 to 11: B12; slopes 12, 13, 14: B13; slopes 15, 16, 17, 18: B14; slope 19: B15; slopes 20, 21, 23, 24: B21; slope 25: BX (absence of detection for slopes 3 and 22).

An examination of this figure shows that the individuals which have the haplotype B12 give the same band and are thus very homogeneous. The same observation applies to the individuals B14. In contrast, with B21, it will be noted that the profiles are different, which shows the inefficiency of the serological approach. In view of the position of the band of BX, it can be established that this is a B4 haplotype.

The practical application of this method involves submitting the naturally resistant individuals to the protocol described above, taking into account the two Rfp-Y and B systems of

the MHC, and selecting from the animals to be tested only those whose profile corresponds to that of the resistant animals.

The invention thus provides the means of verifying the homogeneity of the animals and of carrying out rigorous selections taking into account each system of the MHC and, within these systems, the genes sought.

CLAIMS

1. Nucleic acid molecules, isolated from their natural environment, of genes coding for proteins involved in controlling resistance or susceptibility to the development of tumours in chickens, such as those of Marek's disease, and of regions related to the said genes, characterised in that they have nucleic acid sequences of genes of the B or Rfp-Y system corresponding to the major histocompatibility complex of farmed birds, with the exception of the sequences of class II B-L genes, gene 17.5, gene 12.3 and of the class I B-FIV gene, or are capable of pairing with one of the strands of a gene capable of coding for a protein as defined above under conditions of low stringency.

2. Nucleic acid molecules according to claim 1, characterised in that they correspond to one of the following sequences:

- sequence of the Rfp-Y system
  B-FV (figure 1); B-FVI (figure 2);

- sequence of the B system,
  genomic 8.4 (figure 3); B-FI (figure 4); C121 (figure 5), DM (figure 6), TAP1 (of the beginning of exon 2 at the 3' end) (figure 7) and TAP2G (figure 8).

3. Nucleic acid molecules according to claim 1 or 2, characterised in that they correspond to a part of the sequences defined in claims 1 or 2, this part being specific and discriminating for a given gene of the B and Rfp-Y systems.

4. Nucleic acid molecules according to claim 3, characterised in that they are oligonucleotide molecules corresponding to a part of the polymorphic region of the systems of the major histocompatibility complex of chickens.

5. Nucleic acid molecules according to claim 4, characterised in that they are oligonucleotide molecules corresponding to a part of an exon.

6. Nucleic acid molecules according to claim 4, characterised in that they are oligonucleotide molecules corresponding to a part of the polymorphic region which is not associated with the function of the systems of the MHC, such as microsatellite regions.

7. Method of genotyping farmed birds and particularly chickens, characterised in that it comprises:

- amplification of a sample of nucleic acid originating from the animal to be studied using one or more pairs of primers capable of hybridising specifically with the nucleic acid of a polymorphic region of the Rfp-Y or B systems of the MHC of the said birds, and

- detection of the PCR products obtained.

8. Method according to claim 7, characterised in that the primers are developed from molecules according to any one of claims 3 to 6, and of any gene (and related region) coding for a protein involved in controlling resistance or susceptibility to virus-induced tumours in farmed birds and particularly chickens, particularly class II B-L genes, gene 17.5, gene 12.3 and gene B-FIV.

9. Method according to claim 7 or 8, characterised in that the detection of the PCR products is carried out by sequencing.

10. Unit or kit for genotyping farmed birds and particularly chickens, characterised in that they contain the necessary reagents for carrying out at least one PCR and the detection test, according to the method of claim 8 or 9, particularly the primers developed from the nucleic acid molecules according to any one of claims 3 to 6.

Abstract

The invention relates to nucleic acid molecules which make it possible to detect those genes of the MHC involved in the phenomena of resistance or susceptibility to the development of virus-induced tumours. The primers developed from said molecules can be used in a method of genotyping farmed birds, and particularly chickens, characterised in that it comprises:

- amplification of a sample of nucleic acid originating from the animal to be studied using one or more pairs of primers capable of hybridising specifically with the nucleic acid of a polymorphic region of the Rfp-Y or B systems of the MHC of said birds, and
- detection of the PCR products obtained.

(No drawings)

RFLP type. However, these methods do not provide accurate knowledge about the phenomenon of resistance or susceptibility to disease, particularly due to lack of discrimination with respect to genes of the B or Rfp-Y systems of the MHC.

Various studies concerned the positionning of CMH genes in chicken.

In Guillemot et al's article, EMBO Journal, 1988, Vol.7, N°9, p. 2775-2785, the authors, some of them are co-inventors, disclose class I β genes cartography, in chicken, and describe 5 class II genes (B-L).

In Immunogenetics 1994, 39 : 71-73, the authors disclose homology regions between Rfp-Y and CMH-B genes.

Immunogenetics 1966, 44 : 242-245 concerns the study of the association between Rfp-Y haplotype and Marek's disease in chicken. Said article mentions that a second histocompatibility complex of genes was found in chicken, i.e. Rfp-Y complex which comprises class I and class II CMH genes. But, said both documents do not disclose sequenced genes useful for the genotyping of chicken.

Likewise, in Immunogenetics 1994, 39 : 221-229, the authors, some of them are co-inventors, disclose the link between a new member of the lectin supergene family and CMH genes of chicken.

Eur. J. Immunol 1993, 23 : 1139-1145, in which some co-inventors are also co-authors, discloses 5 B-LB genes in chicken belonging to two different families.

The Journal of Immunology, 1992, Vol. 148, 1532-1546 discloses preliminary, comparative studies concerning BF sequences in chicken.

The work of the inventors on MHC gene sequencing showed the genetic complexity of this region, which led them to take account of another type of polymorphism, namely that based on the sequence of these genes and of related regions, such as those of their promoters and of the microsatellite regions. The inventors thus developed methods for obtaining highly specific oligonucleotide molecules of the polymorphisms observed, making it possible to identify the parts of genes, and even the sites involved in controlling resistance or susceptibility to the development of tumours.

The specific nature of these molecules with respect to a given gene of one of the systems of the MHC makes them particularly reliable discriminating tools for identifying accurately the capacity for resistance or susceptibility of the chicken studied, or of other birds, to a viral infection, and for studying at molecular level the sequences of the MHC involved.

The object of the invention is, therefore, to provide nucleic acid molecules which make it possible to detect specifically, in farmed birds and particularly in chickens, the genes associated with the MHC involved in the phenomena of resistance or susceptibility to the development of virus-induced tumours.